A Novel Paradigm for the Sex Differences in Response to Ultraviolet Radiation Exposure

An Undergraduate Honors Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Bachelor of Science with Distinction in the School of Health and Rehabilitation Sciences of The Ohio State University

Evan Sommer
Undergraduate Biomedical Science Major
School of Allied Medical Professions
The Ohio State University
2015

Thesis Committee:

Advisor: Tatiana Oberyszyn, Ph.D
Amanda E. Toland, Ph.D
Amanda Agnew, Ph.D

Abstract

Skin cancer is the most common cancer in the world. Its main cause is chronic ultraviolet (UV) radiation exposure, responsible for initiation of DNA damage and cancer-enhancing inflammation. Relative to females, males are up to three times more likely to develop skin cancer and seventy percent more likely to die from it once affected. Our goal is to find causes of these significant sex differences. Given data from previous studies that demonstrates sex differences in the levels of inflammatory markers and immune cells, we hypothesize that sex differences in the inflammatory response to UV radiation is the causative factor of the sex differences in skin cancer. We showed that females demonstrated higher levels of inflammatory cytokines both in the skin and serum relative to males following chronic irradiation, and that they also have greater inflammatory edema and myeloperoxidase levels acutely, providing evidence of this inflammatory difference. We also show that circulating testosterone reduction from castration seems to play little role in these differences, while circulating estrogen reduction following ovariectomization results in lesser inflammation, correlating with a greater tumor burden in ovariectomized females from chronic studies in the context of our hypothesis.

Table of Contents

Introduction

Skin cancer is by far the most common form of cancer in the world today. It has a higher incidence than all other cancers combined and between the years of 1992 and 2006, a 77% increase in skin cancer treatment was recorded, making it one of the fastest growing by number of cases[1,2]. In the United States, it is estimated that one in every five people will get skin cancer in their lifetime, creating a significant need for research into causes and beneficial treatments[3].

Broadly, there are two different classifications of skin cancer: Melanoma, and Non-melanoma Cancers (NMSC). The Oberyszyn lab focuses on Non-melanoma, which includes basal and squamous cell carcinomas (SCC). Each year there are an estimated 700,000 new cases of SCC in the United States alone, making it the second most common form of skin cancer behind basal cell carcinoma, which has an incidence of 2.8 million new cases per year[4,5].

The largest cause of skin cancer is Ultraviolet (UV) radiation from the sun, and more recently, tanning beds[6]. Ultraviolet radiation results in skin cancer through the creation of mutations in the DNA of exposed cells. UV radiation excites electrons of adjacent thymine nucleotides, causing them to bond together, resulting in thymine dimers. When these mutations occur on specific loci of the genome - the most notable of which for SCC is the p53 tumor suppressor gene which serves as the site of transcription for the p53 protein, a regulator of the G_1 phase in the cell cycle –cell proliferation is deregulated, resulting in a benign tumor[7] which can progress to SCC with further UV exposure.

An accepted model for the study of UV radiation induced skin cancer is the hairless skh-1 mouse model[8,9,10]. These mice are hairless, making them convenient in the study of UV carcinogenesis as radiation is more easily delivered directly to the skin without the need to shave the animals. The skh-1 mice also have a deficiency in melanocytes. These cells normally serve to produce melanin, a pigment which protects the skin from radiation[11]. As such, a deficiency in these cells causes more rapid tumor development, which allows for less lengthy studies.

In most cases, the body's response to cellular damage such as that from UV radiation creates an inflammatory response that serves to recruit phagocytic immune cells to the affected area. These phagocytic immune cells, which include neutrophils, monocytes, and macrophages, engulf damaged and mutated cells to prevent them from replicating uninhibited, thereby preventing tumorigenesis[12]. This is the body's systemic response to ultraviolet radiation exposure.

Several key cytokines are known to play important roles throughout the inflammatory process. Some of the most important and well-studied include Tumor Necrosis Factor alpha (TNF-a), Interleukin 6 (IL-6), Interleukin 1 (IL-1), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), and Interleukin 16 (IL-16) Broadly, these cytokines serve as chemoattractants—which recruit immune cells to affected areas—effectors of temperature change such as that seen in fever, or stimulators of immune cell maturation to increase immune surveillance in the presence of potentially harmful agents in the cellular environment[13, 14, 15, 16].

Another effect of the inflammatory response is an increase in cutaneous edema. Generally, edema is localized swelling in tissues, which results from fluid and plasma delivery and retention in the affected area. Inflammation results in increased blood vessel permeability allowing plasma containing immune cells and other inflammatory agents to reach tissues which need them[17]. As a direct effect of inflammation, edema can be used as a marker of the inflammatory response.

Another marker of inflammation is Myeloperoxidase (MPO), which is largely produced by neutrophils. Neutrophils are the most abundant type of white blood cells, and during the initial inflammatory response they follow signals from chemoattractant cytokines to infiltrate tissues containing potential threats to the cellular environment. As phagocytic immune cells, they can engulf harmful agents like bacteria. Using hydrogen peroxide as an oxidizing agent, MPO is a peroxidase enzyme that allows neutrophils to produce cytotoxic acids and free radicals that then kill the engulfed agents. Neutrophils are also capable of releasing these cytotoxic agents out into the cellular environment to destroy potential threats[18]. This, however, can also lead to off target damage to healthy cells, and has been shown to increase the risk of cancer in a chronic setting[19]. Multiple recent studies have shown that even with these risks following chronic exposure, activated neutrophils can target and limit proliferation of tumor cells[20].

The enormous number of new skin cancer diagnoses each year is not shared equally between the sexes. Non-Melanoma Skin Cancer prevalence has been shown to differ significantly between male and female populations, with males developing 2x as many basal cell carcinomas, and 3x as many squamous cell carcinomas, relative to females. Males are also

up to 70% more likely to die from skin cancer once affected[21, 22]. Our lab has previously shown that this difference is not simply due to increased exposure or decreased protection in males compared to females. Data from studies in which male and female Skh-1 hairless mice, when exposed to equal UV doses, showed a significantly higher incidence of skin tumor development in male mice[8]. This significant sex difference in the most common cancer in the world demands investigation so that treatment can be delivered to those at high risk. Interestingly, there were also significant differences in the inflammatory response between the sexes with males having a decreased UV mediated inflammatory response compared to males [8]. Due to the relatively large inflammatory response to UV irradiation, it is possible that differences in immune cell levels and inflammation between the sexes contribute to differences in skin tumor development.

As the mediators of inflammation, immune cells are of great interest. Once cell type in particular, myeloid derived suppressor cells (MDSCs) are known for their roles as regulators of the immune system, suppressing the activity of T-cells. By suppressing immune activity, MDSCs have effects dissimilar to those of cells like neutrophils. Flow cytometric analysis of splenic tissue samples revealed that relative to females, males demonstrate 55% higher levels of splenic Gr-1$^+$ CD11b$^+$ myeloid suppressor cells[23]. This evidence highlights the idea that within the same species, sex differences in immune cell levels can exist.

Over recent years, the idea that adipose tissue simply serves as a site for energy storage in the body has lost ground to the idea that it is actually immunologically active. The presence of immune cells in adipose tissue has been confirmed and it has been shown that the various adipose tissue depots contain varying levels of different immunological cell types[24]. In the

United States today, where obesity rates continue to hold at epidemic levels (around 35% of all Americans are classified as obese[25]), the implications of this idea are significant.

Adipose tissue has been implicated in other cancers through the release or suppressed release of certain cytokines into the bloodstream. The most significant effects of these cytokines with regard to cancer progression include contribution to the creation of a chronic inflammatory state, stimulation of angiogenesis, and suppression of apoptosis[26, 27]. Given these implications, combined with the relatively novel idea that immune cells can be found in the adipose tissue, and our own evidence for sex differences in immune cell levels and adipose response to UV radiation exposure, there is potential for various types of immune cells within gonadal adipose tissue and the cytokines they secrete to present a link between adipose tissue and the body's inflammatory response to UV radiation exposure.

With clear demonstration of differences in the development of skin tumors between the sexes combined with evidence of sex differences in immune cell levels and in the inflammatory response, the goal of this senior honors thesis is to confirm the presence of these inflammatory differences, and investigate their causes. We hypothesize that differences in the intensity of the inflammatory response between males and females are responsible for the creation of the sex differences in skin cancer development. Given the function and effects of inflammation in the body, these inflammatory differences likely influence tumor development through pre-malignant cell destruction and/or chronic inflammation.

Materials and Methods

Role of Adipose Tissue in UV-mediated inflammation

All tests for the determination of the role of adipose tissue in the systemic response to UV radiation exposure were completed on gonadal adipose tissue harvested post sacrifice from mice which received controlled doses of UV radiation (2240J/m^2) three times weekly for ten weeks, and were sacrificed after 25 weeks following tumor development. The adipose tissue was either flash frozen in liquid nitrogen, or was fixed in formalin for two hours following harvest to preserve protein structure. Mice were from one of four groups, which included male and female UV irradiated or non-irradiated mice.

Morphological Testing of Gonadal Adipose Tissue

Morphological tests of gonadal fat were conducted to test for differences in adipocyte size between the groups. Changes in adipocyte size have been implicated in altered adipokine secretion which has the potential to influence inflammation [40]. Formalin fixed paraffin embedded (FFPE) adipose tissue specimens were sectioned at thickness of 10um on a microtome for observation under light microscopy. These microtome sections were stained with hematoxylin and eosin (Richard Alan Scientific) and images of representative areas of each section were captured and analyzed in ImageJ analysis software. Using the formula for the area of an ellipse (Area = πab), average cell areas were determined by measuring perpendicular axes of the adipose cells, which were approximately elliptical. Average cell areas were compared between test groups.

Immunohistochemical Analysis of CD45+ Immune Cells

Immunohistochemical (IHC) analysis of gonadal fat tissue was performed, with the goal of locating and quantifying the various types of immune cells and comparing their levels between the sexes and different UV exposure groups. With our first tests, we sought to identify the presence of CD45, a leukocyte common antigen [28], in the adipose tissue as a broad test for various types of leukocytes.

Ten micron sections of FFPE adipose tissue were sectioned as discussed above. The slides were heated at 60 degrees Celsius for 30 minutes to soften the paraffin, and the slides were simultaneously de-paraffinized and rehydrated in a series of baths in Clear-rite organic solvent (Richard Alan Scientific), 100 percent ethanol, 75 percent ethanol, 50 percent ethanol, and water. Each tissue sample was circled with Immedge pen (Vector Labs) to keep reagents on top of the tissue in later steps. A peroxidase block was performed in 3 percent hydrogen peroxide (Fisher Scientific) for 10 minutes at room temperature followed by a bath in Phosphate Buffered Saline with 1% Tween-20 (PBST; Bio-Rad). Antigen retrieval was performed by submerging the sections in Antigen Retrieval Solution (Vector Labs) and microwaving for 2-5min intervals in the microwave at 100% followed by 5min at 50%. Slides were then cooled for 20min at room temp. Non-specific binding was blocked for 15min each with Avidin and Biotin blocking solutions followed by 30min with 10% casein (Vector Labs). The samples were then incubated with a rat anti-mouse CD45 primary antibody (Abcam) for binding to the CD45 antigen at 4°C overnight. Slides were then incubated for 30min with a biotinylated rabbit anti-rat IgG secondary antibody (Vector Laboratories). The samples were then incubated with ABC elite (Vector Laboratories) for 30min followed by DAB solution (Vector Labs) for 10min. In

between steps, slides were washed 3x for 5min with PBST. The slides were then cover slipped and visualized via light microscopy. Splenic tissue was used as a positive control to test for proper function of the antibody, as splenic tissue is known to have a high concentration of leukocytes.

Protein Arrays

A series of 8 adipokine and 8 cytokine arrays (Adipokine and Cytokine Proteome Profiler Array Panel A (R&D Systems) were performed to determine if the secretion of various factors either by the adipose tissue or the immune cells within the adipose tissue could be contributing to the chronic inflammatory response to ultraviolet radiation exposure and/or the sex differences in this response. Each array was made up of antibodies specific for one of forty different proteins spotted onto a nitrocellulose membrane.

The arrays were incubated with either serum (cytokine and adipokine arrays) or protein that had been previously extracted from gonadal fat (adipokine arrays only) that had been flash frozen following sacrifice of chronically irradiated male and female mice. Following a blocking procedure outlined in the manufacturer instructions, 100uL of serum or 300ng of protein, was incubated overnight at 4°C. Each membrane was washed with the included wash buffer for 30 minutes at room temperature, then incubated with Streptavidin-HRP for 30 minutes at room temperature. Each array was then incubated with 2mL of chemiluminescent reagent for 5 minutes at room temperature and photographed. The relative levels of each adipokine were determined from the array images using ImageJ analysis software.

The Effects of Endogenous Testosterone on Tumor Development

To determine the effects of circulating testosterone on tumor development, a castration study was completed. Following the delivery of a standard dose of 2240J/m^2 of UV radiation three times weekly for 15 weeks, one group of hairless skh-1 mice was castrated, thereby removing the predominant producer of endogenous testosterone[29]. This was done to simulate a midlife drop in testosterone levels seen in human males. Another group of mice received a sham surgery to control for the inflammatory effects of surgery, but retained their testes. Tumor burden, defined for skin cancer research as the total skin area occupied by tumors, was determined along with tumor number over the 10 weeks of tumor development following surgery.

The Effects of Endogenous Estrogen on Inflammation

Mouse Model

Three groups of mice were given one dose (2240J/m^2) of UV radiation. One group of female mice was ovariectomized 2 weeks prior to irradiation, thereby removing a large concentration of endogenous estrogen. Each of the two other groups, one female and one male, received sham surgeries to control for the inflammation caused by the surgery itself. Skin fold thickness was measured with calipers as a marker of inflammation prior to irradiation, and following sacrifice [30, 31]. In each group, 5 mice were sacrificed 24 hours following irradiation, 5 after 48 hours, and 5 after 1 week, for a total of 15 mice per group. These sacrifice intervals allowed for comparing peak inflammation and also partial resolution of the inflammation

between the groups. Comparisons were made between the female and male sham surgery mice to demonstrate the baseline difference in inflammatory response between the sexes. By comparing the female sham mice to those that were ovariectomized, analysis of the role of endogenous circulating estrogen in the inflammatory response was completed. Following sacrifice, skin, serum, and gonadal, inguinal, and subscapular fats were harvested for later testing. Ten mm punch biopsies of the skin were flash frozen in liquid nitrogen to complete MPO assays.

Skin Fold Thickness

Skin fold thickness was measured in each mouse 2 weeks following surgery, prior to UV irradiation and then again just prior to sacrifice. As a marker of edema from inflammation this test allows for the determination of the relative levels of edema between mouse groups[30, 31]. Using forceps to gently pinch and lift the skin into a fold, calipers could then be used to measure the width of this fold in millimeters. A greater width corresponds to more edema. As each group contained mice that were sacrificed at 24 hours, 48 hours, and 1 week time points, we were able to obtain data on the change in edema over time in response to irradiation.

Myeloperoxidase Assays

As previously stated, myeloperoxidase is a peroxidase enzyme that uses hydrogen peroxide as an oxidizing agent to produce cytotoxic hypochlorous acid and free radicals[18]. 10mm punch biopsies of skin from the dorsum of mice had been flash frozen during sacrifice. These skin samples were homogenized in a solution of 5%

Hexadecyltrimethylammonium bromide and potassium phosphate buffer, followed by 3 cycles of sonication, flash freezing, and then thawing to further break down the tissue and cause cell lysis. Following cell lysis, the samples were centrifuged at 13,000xg for 15 minutes, and the protein containing supernatant was removed into separate tubes. This supernatant (10uL) was mixed with a substrate solution containing hydrogen peroxide in wells of a 96 well plate. As MPO is a peroxidase enzyme, incubation with hydrogen peroxide results in a colorimetric reaction. The brown color produced by the reaction was read by the SpectraMax plate reader over the course of 5 min. Sample concentrations of MPO were determined by comparing absorbance values of the samples to those of the MPO standard curve.

Role of Topical Estrogen in Chronic UV-Mediated Cytokine Production

Cytokine Arrays

Serum and skin were harvested from skh-1 mice of 4 separate groups. One group contained male mice who were exposed to a standard dose of 2240J/m^2 of UV radiation three times weekly for 15 weeks, followed by 10 weeks of tumor development during which they were treated topically with 10nmol 17-β-estradiol.. The second group of males underwent the same UV irradiation schedule, but received only acetone during tumor development. Acetone was the vehicle for topical estrogen delivery and is therefore a control in this context. The remaining two groups went through identical treatment schedules but were females. Cytokine expression profiles in serum and skin of 2 mice in each group were analyzed via Proteome Profiler Panel A cytokine arrays from R&D Systems as described above. Skin was homogenized in NP-40 buffer, subjected to 3 cycles of sonication/freezing/thawing, incubated on ice for 1

hour, and centrifuged at 13,000xg for 15min. The concentration of total protein in each supernatant was determined by BCA, and each array was incubated with a 300ug of skin protein. Past this point, the protocols for both serum and skin arrays was identical. Refer to the adipokine array procedure previously detailed.

Results

Gonadal Adipose Tissue

Representative photographs of adipose tissue from unirradiated female (top left), male (top right) and UV exposed female (bottom left) and male (bottom right) mice demonstrate the adipose morphology. Average area analysis of these pictures demonstrated no appreciable differences between the sexes or UV treatment groups (Figure 1B). It was shown that male UV mice had an average cell area of 1835µm^2, male control mice who did not receive UV irradiation demonstrated an average cell area of 2446µm^2, female UV mice had an average cell area of 2346µm^2, and female non-irradiated control mice demonstrated an average cell area of 1843µm^2.

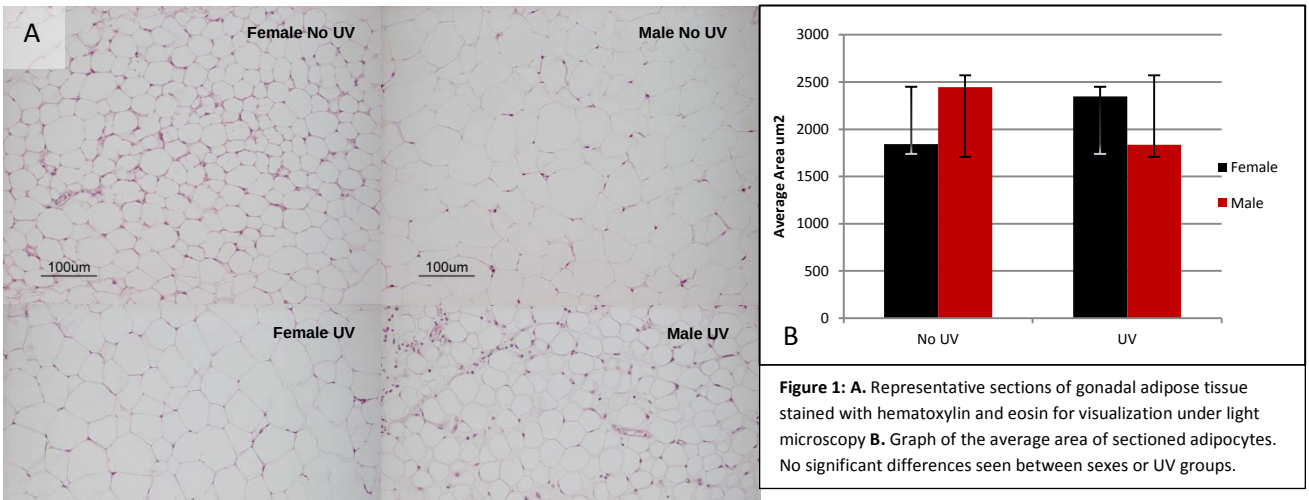

Figure 1: A. Representative sections of gonadal adipose tissue stained with hematoxylin and eosin for visualization under light microscopy **B.** Graph of the average area of sectioned adipocytes. No significant differences seen between sexes or UV groups.

Infiltration of CD45^{+} Immune Cells into Gonadal Adipose Tissue following UV exposure

Figure 2 is a representative image demonstrating CD45+ cells in adipose tissue. Quantification of the number of CD45^{+} immune cells within the gonadal adipose tissue between sexes and UV groups did not reveal any significant differences (data not shown).

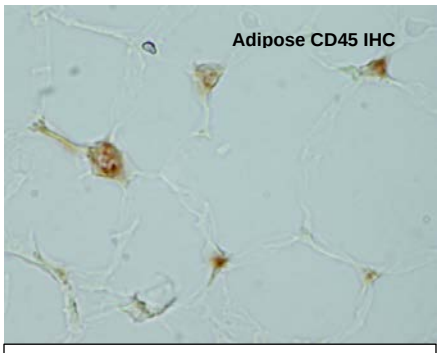

Figure 2: an example of the maximum number of cells visualized in CD45 IHC stained adipose tissue samples

Alterations in Adipokines/Cytokines Following UV Exposure

Some interesting trends were seen in the levels of adipokines and cytokines between the sexes and UV groups. Figure 3 is a representative image of a protein profiler Array. Figure 4 demonstrates the relative levels of serum cytokines (Figure 4A), skin cytokines (Figure 4B), serum adipokines (Figure 4C), and gonadal adipose tissue adipokines (Figure 4D) in both female and male UV-exposed mice as a ratio of those values in no UV control mice. In general, females demonstrated a greater positive difference in pro-inflammatory markers from no-UV to UV compared to females. The data highlighted interesting trends in the inflammatory response to UV irradiation between males and females that warranted further exploration by additional testing.

Figure 3: A representative image of a protein profiler array

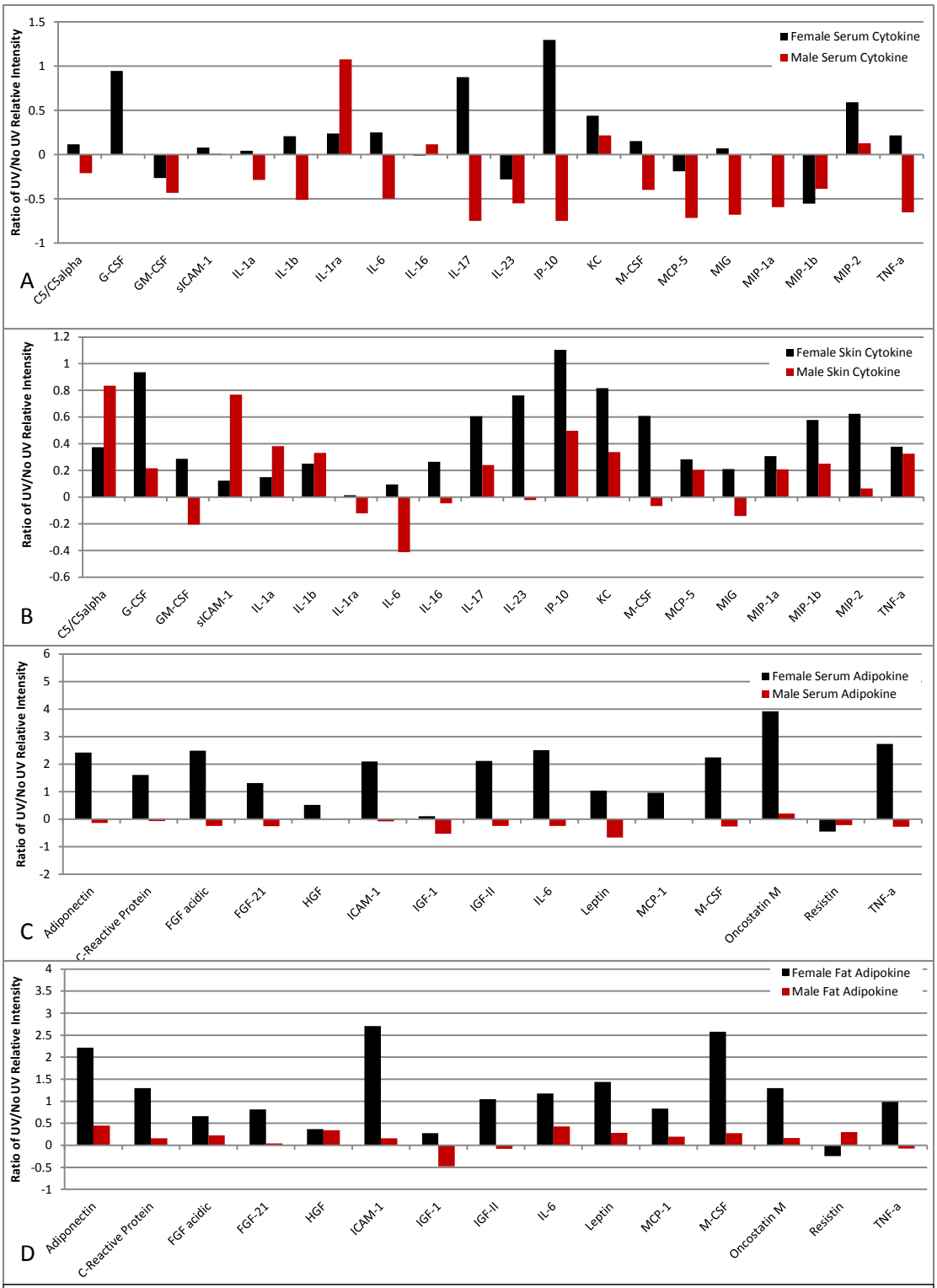

Figure4: A.. A graph of the ratio between UV irradiated and non-irradiated mice for each serum cytokine **B.** A graph of the ratio between UV irradiated and non-irradiated mice for each skin cytokine. C**.** A graph of the ratio between UV irradiated and non-irradiated mice for each serum adipokine D**.** A graph of the ratio between UV irradiated and non-irradiated mice for each adipose tissue adipokine. Graphs were used to compare differences in inflammation between UV and no-UV groups in each sex

Effect of Castration on Tumor Development

It was seen that castration seemingly had no significant effect on tumor number (Figure 5a) or tumor burden (figure 5B). Mice that underwent 15 weeks of chronic UV irradiation and were then castrated to remove the major producer of endogenous testosterone showed an average tumor number of 8.2 and an average tumor burden of 126.66mm^2. Those who underwent the same irradiation schedule but only received a sham surgery demonstrated a tumor number of 10.25 and a tumor burden of 212.61mm^2.

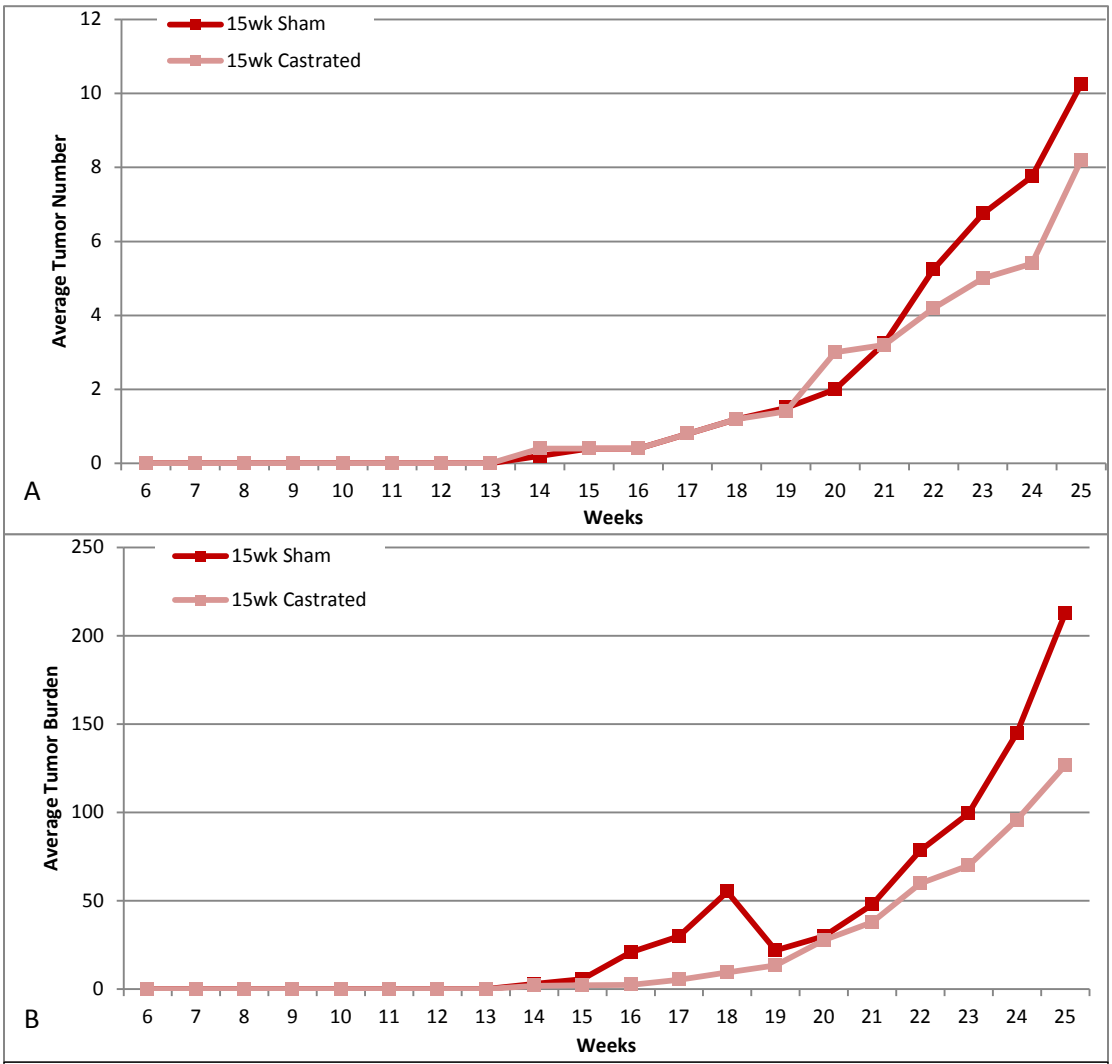

Figure 5: A. Graph of average tumor number per mouse following 15 of UV irradiation and either castration or sham surgery. **B**. Graph of average tumor burden per mouse following 15 of UV irradiation and either castration or sham surgery.

Effect of Ovariectomy on Acute UV-mediated edema

Skin fold thickness is a measure of the extent of edema from inflammation over time[30, 31]. As shown in the graph in figure 6, prior to irradiation males demonstrated higher skin fold thickness than both groups of females, with an average value of 0.74mm for the males compared to values of 0.64mm for intact and 0.58mm for the ovariectomized females. At the 24 hour time point we see that males again have higher skin fold thickness than both groups of females. However, all groups show an increase in skin fold thickness compared to pre-irradiation levels and it should be appreciated that both groups of females had a greater change from baseline levels relative to males. Males demonstrated an average change of +0.28mm, while the sham females changed by an average of +0.3mm and the ovariectomized females changed by +0.395mm on average. At the 48 hour time point, it is clear that both sham and ovariectomized females surpass the levels of skin edema demonstrated by the male mice, with thicknesses of 1.26mm, 1.3mm, and 1.12mm, respectively. At the 1 week time point we see that male mice demonstrate a greater skin thickness (0.92mm) than intact (0.78mm) and ovariectomized (0.73mm) female mice.

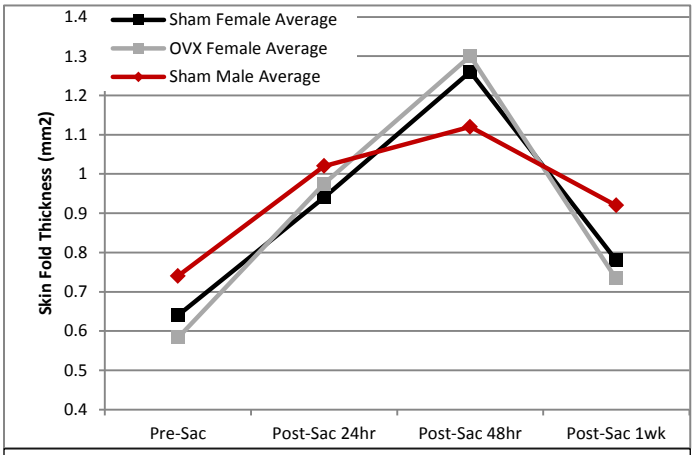

Figure 6: Skin fold thickness at all time points

Effect of Ovariectomy on Myeloperoxidase Activity

As previously mentioned, MPO serves as a marker for inflammation through its role as a peroxidase enzyme released by activated neutrophils[18]. As shown in Figure 7, female mice have an average MPO value of 0.0128 which is more than double that seen in male mice. While not significantly different, the ovariectomized females lie in-between these values at 0.0109. At 48 hours following irradiation, all of the groups demonstrated an increase in MPO relative to their 24 hour values, but the trends between the groups remain consistent. Males demonstrate the lowest values at 0.0118, while the sham females demonstrate the highest at 0.0153. The estrogen deficient ovariectomized females have an average MPO value of 0.0142, which lies in-between the intact males and females. At the 1 week time point, MPO levels decreased in all groups, dropping below their 24 hour levels as inflammation was resolved. Again, because we were unable to determine the MPO levels prior to irradiation, it is impossible to comment on the extent of inflammatory resolution relative to baseline levels. At 1 week, sham females again demonstrated the highest average MPO value at 0.0047. At this time point, however, the ovariectomized female value of 0.0029 was below that of males, which was 0.0032.

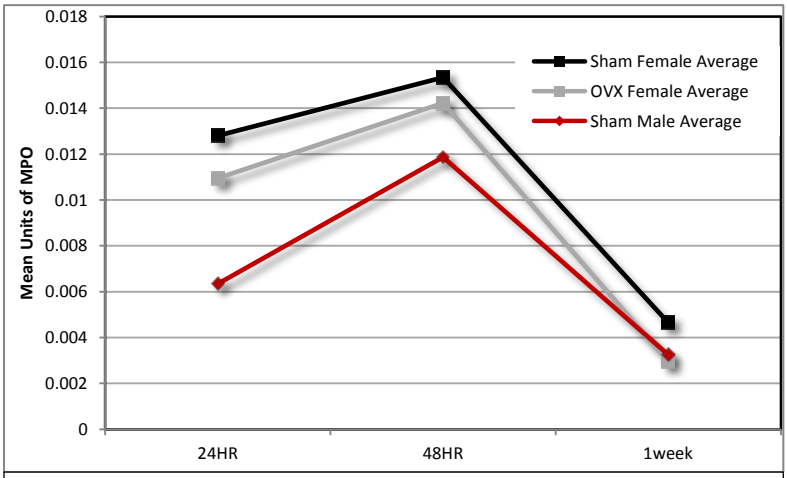

Figure 7 A graph of the average mean units of MPO per mouse in each group

Role of Topical Estrogen in Chronic UV-Mediated Cytokine Production

Preliminary data in our lab suggests that exogenous application of estrogen to male mice may be protective (data not shown) suggesting that cutaneous estrogen may regulate the inflammatory response to UV. In order to examine this, the levels of various cytokines involved in the inflammatory response and immune system were determined in the skin of chronically irradiated male and female mice following topical estrogen delivery, and were compared to those of mice that received no topical estrogen. Out of the 40 proteins tested, 8 were present in high enough concentrations to be reliably measured with image analysis software. The levels of each visible protein in each mouse group are relayed in figure 8a, and the difference in levels between groups given topical estrogen and those that were not are illustrated in figure 8b. It

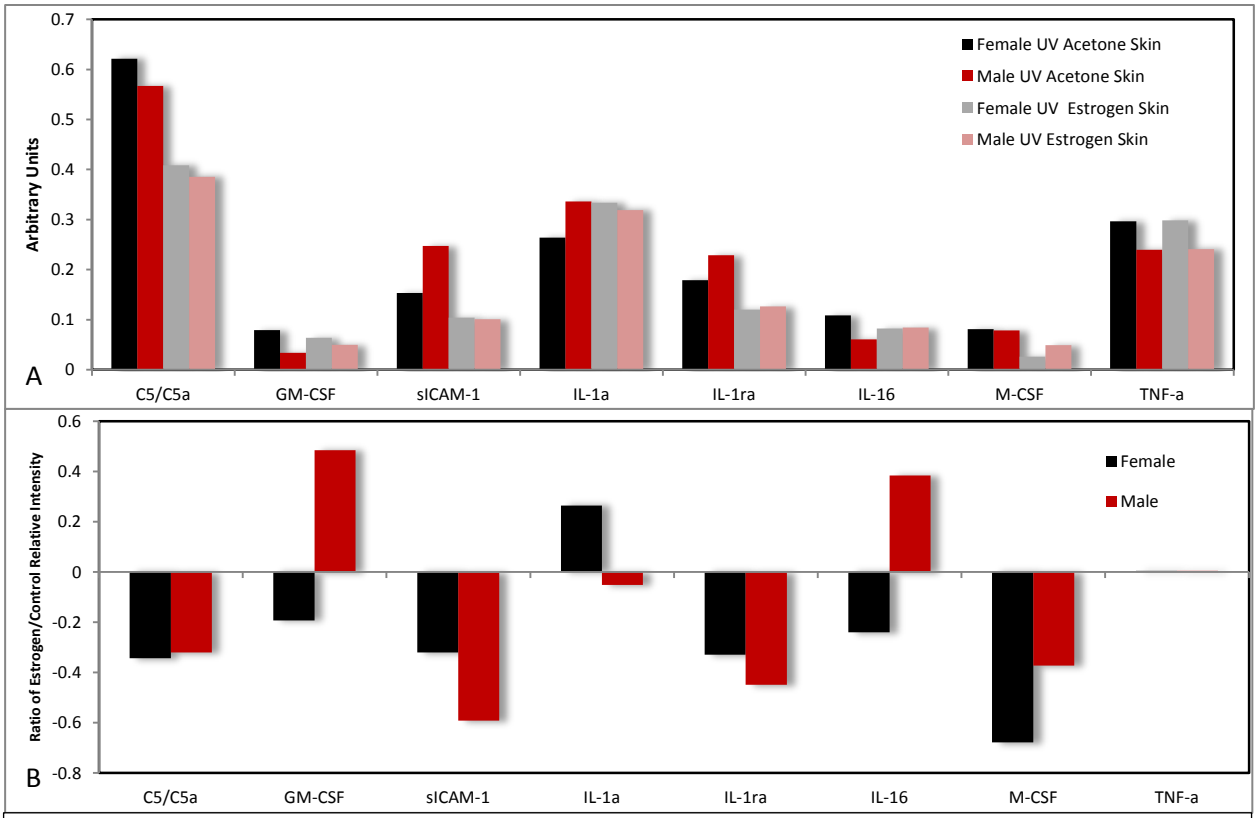

Figure 8: A. Graph of the levels of several skin cytokines with a known important role in inflammation. **B.** Graph of the difference between topical estrogen and control treatment groups seen in (A) for comparison of the sex differences in response to UV irradiation.

was seen, for instance, that in the control mice females had higher levels of IL-16—a chemoattractive cytokine—relative to males [16]. Following topical estrogen delivery female levels of IL-16 were lower compared to control mice, while males given topical estrogen demonstrated higher levels than their control counterparts.

Discussion

When first starting to explore the potential existence and causes of inflammatory differences between the sexes, our lab had recently found some studies showing a role for adipose tissue in the development of skin tumors [32]. Upon completing some very preliminary studies showing a correlation between gonadal adipose tissue weight and skin tumor burden (data not shown), we thought the novelty of the subject, combined with its implications in a generally obese society demanded investigation. Planning to investigate the differences in adiposity, adipose immune cell levels, and adipokine/cytokine secretion into systemic circulation, we sought to identify trends between the sexes in response to UV irradiation that would highlight a potential role of adipose tissue in the inflammatory response. Neither morphological testing, nor tests for the levels of immune cells delivered significant differences, but tests of the levels of systemically secreted adipokines and cytokines revealed interesting differences between the sexes.

Previous research in our lab demonstrated that inflammation is a critical component to UV induced skin cancer development in female mice [9, 10]. Male mice have a dampened inflammatory response to acute UV exposure but have increased tumor development compared to females (Thomas-Ahner). However, no studies have examined sex differences in inflammatory markers of tumor bearing mice. Using protein array technology designed to identify proteins of interest for future study, we examined the levels of inflammatory cytokines in both male and female chronically irradiated serum and adipose tissue. Broadly, UV exposure triggered a sustained increase in pro-inflammatory mediators in females compared to males.

This provided us with some initial evidence that the chronic inflammatory milieu could differ between the sexes. We were not confident that this difference was caused by the adipose tissue, however, as the morphological testing and determination of immune cells levels revealed no differences. It is likely then, that any differences in the intensity of inflammation between the sexes are caused by a factor other than the adipose tissue, and that the trends seen in adipokine secretion are secondary effects. Further testing sought to identify other factors.

Previous studies have suggested that castration of either male or female mice may affect chemically induced skin cancer development[42], however no studies have determined the role of castration in UV-induced skin carcinogenesis. Unpublished studies previously conducted in our lab were designed to investigate the effects of depressed and elevated levels of estrogen on tumor development. After 15 weeks of UV irradiation, female mice were separated into four groups. The first group was ovariectomized to remove the primary producer of endogenous estrogen, corresponding to a decrease in estrogen in post-menopausal patients. Mice of another group were left intact, receiving sham surgeries to control for the inflammation caused by the procedure itself. Another group received sham surgeries as well, but was also given daily topical estrogen treatment, which served to increase the levels of estrogen localized within the skin. The last group was ovariectomized and given topical estrogen, modeling a decrease in circulating endogenous estrogen combined with direct delivery of exogenous estrogen to the skin. Intact mice given topical estrogen and those who underwent ovariectomy without topical estrogen treatment developed more tumors compared to intact control treated mice suggesting that both a reduction in endogenous estrogen or an increase in exogenous

estrogen after chronic irradiation correlated with an increased tumor burden. These studies suggest that systemic estrogen may impart protection against skin cancer development in female mice.

In order to assess the effects of testosterone in skin tumor development, we completed a castration study. Two groups of mice were irradiated for 15 weeks, at which point one received sham surgeries while the other was castrated to remove the major producer of endogenous testosterone [29]. After tumor development was measured and tracked for a total of 25 weeks, no significant differences in skin tumor number or burden were shown between the groups, leading us to believe that systemic testosterone plays little role in the sex differences in skin tumor development. Taken together with the ovariectomy studies, we believe estrogen may play an important role in the observed sex differences in skin cancer. This interpretation, combined with evidence from the adipose tissue testing of levels of inflammatory markers led us to believe that estrogen in some way interacts with the immune system to create a more robust inflammatory response. The basic goal of inflammation is to respond to potential threats to a normal cellular environment, eliminate those threats, and then return to baseline levels to prevent off-target damage from chronic irradiation [12]. In this context, it is possible that a more robust inflammatory response to cellular damage caused by UV irradiation serves to rid the body of more pre-malignant cells, thereby reducing the risk that damaged cells acquire a dysregulation in proliferation and form skin tumors. Our next group of experiments sought to first demonstrate the existence of a more robust and/or sustained acute inflammatory response to UV irradiation in females, and then provide evidence for the implication of estrogen in this inflammatory difference.

Skin fold thickness was determined as a marker of edema-creating inflammation [30, 31]. As previously mentioned, males demonstrated higher values of skin thickness prior to irradiation, and at both the 24 hour and 1 week time points. At the 48 hour peak inflammatory time point, both groups of female mice showed signs of higher levels of edema. With a broad scope looking first at the differences between males and females, we see that at both 24 hours and 48 hours females demonstrate a greater level of change from baseline relative to males. Because the data points at 24 and 48 hours represent the levels of edema created by acute inflammation, we have evidence that confirms females have higher levels of edema-creating inflammation in response to UV irradiation.

Of further interest is the data at the 1 week time point, showing that males remain farther from their baseline skin thickness levels relative to females. This sustained edematous response could allow for off-target damage from cells like neutrophils that release cytotoxic factors such as hypochlorous acid and myeloperoxidase. During sterile inflammation such as that from UV exposure, the presence of these factors could harm normal, healthy cells, resulting in oxidative DNA damage. We have previously shown that acute UV exposure induces higher levels of oxidative DNA damage in males compared to females (Thomas-Ahner). With males having demonstrated higher skin fold thickness a full one week following irradiation, there is potential for an increased tumor burden in males as a result of failure to regulate inflammation properly after cellular threats have been cleared.

To further investigate sex differences in inflammation we examined MPO activity between the sexes. Male mice produced much less inflammatory MPO than females at all time points. In the context of our hypothesis these trends suggest a more robust and sustained

inflammatory response in females relative to males. This heightened inflammatory response in females, relative to males, correlates with decreased tumor burden from chronic studies where, again, females were shown to develop a lesser tumor burden compared to males. There were some issues with this data, however, in that statistical significance was not achieved at the 48 hour and 1 week time points. In part, this was due the low number of mice in each group. With only 5 mice, any variation in MPO values between them had a large effect on the average and created a greater spread in the standard deviation. Taken together with skin thickness data and MPO demonstration from past studies, we believe that although not statistically significant at 48 hours and 1 week, these trends demonstrate a biologically interesting phenomenon which highlights inflammation as a primary mediator of the sex differences in skin tumor development.

To determine if endogenous estrogen is responsible for the increased inflammatory response in female mice, we compared markers of inflammation between ovariectomized and intact female mice. Skin thickness data was compared between these groups to gauge the relative levels of edema. We saw that at each time point, average skin thickness values were only marginally different between sham and ovariectomized females. The levels of MPO, however, showed some interesting trends. At each time point, intact females demonstrated higher average MPO values relative to the estrogen deficient ovariectomized mice. As a marker of neutrophil activation during inflammation, these MPO trends seem to suggest that reducing the levels of endogenous estrogen leads to a lesser inflammatory response. And in the context of our hypothesis, these lower levels of inflammation correlate with chronic UV studies in which ovariectomized female mice had higher tumor burden than intact mice. To our knowledge, the

idea that inflammation could be protective in the setting of UV mediated skin carcinogenesis is novel. Our data certainly suggests that further studies need to be completed in order to determine the mechanism of protection.

We found it interesting that the levels of MPO differed between the sham surgery and ovariectomized female groups in all time points, while the measured skin fold thickness values did not. It is likely that, instead of interacting with systems which control the amount of plasma displaced within cutaneous tissue during the formation of edema, it is likely that estrogen influences the levels of inflammatory cytokines and immune cells contained within the fluid and tissue. In this way, the same volume of fluid can be displaced into the tissue, while the concentration of cytokines and immune cells contained within it lead to the data seen. Skin thickness is not altered in response to a reduction in endogenous estrogen, but sham females are able to exhibit a higher level of cutaneous MPO. This potential for estrogen to mediate the recruitment and production of higher levels of immune cells and cytokines is further explored in a later part of the discussion.

Having demonstrated a higher level of skin thickness in males one week following irradiation, it is of further interest to us that trends suggest a higher level of MPO in sham females relative to males and estrogen deficient females. Again here, it is possible that the difference in edema and MPO activity can be explained either by an instance of a higher concentration of MPO-creating cells in the sham females, or by the possibility that cytotoxic inflammatory factors other than MPO are present in males. The latter of these possibilities would mean that these other cytotoxic factors would exhibit higher levels in males at a one week time point, corresponding to the higher level of edema demonstrated. We had previously

mentioned the possibility that a failure of male edema to regulate back to normal levels may provide some very basic evidence that males broadly fail to regulate their inflammation after the initial threat had been removed. However, when the skin thickness data is taken together with MPO data, the evidence is unclear and we can therefore neither confirm nor deny the presence of this phenomenon.

Because we hypothesize that a more robust inflammatory response gives some benefit with regard to ridding the skin of pre-cancerous cells, and we have demonstrated higher levels of pro-inflammatory cytokines in serum and adipose tissue of female compared to male tumor bearing mice, it follows that changes in inflammation within the skin have potential to cause changes in skin tumor development. To examine this, we analyzed relative levels of a large panel of pro-inflammatory cytokines in the skin and serum of both male and female mice exposed to topical estrogen following chronic UV exposure. This experiment was largely exploratory in nature; our goal was to identify the presence of potential trends that would be verified in future studies. As such, although statistical significance would be welcomed, it was not expected given that each group had only two representatives. Even so, trends were observed that, when viewed in light of each cytokine's known role in the body, were very interesting.

Complement Component 5a (C5a) is released along with C5b as a product of the cleavage of complement component C5. C5a has a large number of pro-inflammatory roles including chemoattraction and triggering mast cell degranulation to release histamine and Tumor Necrosis Factor Alpha (TNF-a)[33]. These functions mean that higher levels of this cytokine could serve as a marker of higher levels of inflammation in response to UV irradiation.

We saw that in control mice, females demonstrated slightly higher levels of TNF-a than males (figure 8a). In the groups given topical estrogen, this trend between the sexes continues, but both males and females show lower C5a levels relative to the controls. These trends, which again were not statistically significant, may suggest interplay between estrogen and the Complement component cascade of inflammation.

Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) is a cytokine secreted by macrophages, T cells, mast cells and endothelial cells that stimulate the expansion of other granulocytes and monocytes [34]. These cells can then mature into macrophages and dendritic cells to aid in ridding the cellular environment of threats as part of the inflammatory response [35]. Detecting the levels of this cytokine is helpful in that it can give information on the level of immune cell maturation signaling happening in the cutaneous environment in response to irradiation. In our tests, control females had higher levels of GM-CSF relative to males. This highlights the possibility that females have greater levels of immune cell maturation, which could lead to greater immunosurveillance and a more rapid and robust inflammatory response when pre-cancerous cells are detected. In mice treated with topical estrogen, we see again that females demonstrated higher levels than males, but of more interest is the difference in each sex between control and estrogen treated mice. In females, the levels of GM-CSF is lower in mice treated with topical estrogen, while in males, mice treated with topical estrogen have higher levels of the cytokine. This provides evidence that estrogen may in some way interact with this growth factor; in females who already have a high level of endogenous estrogen at baseline, adding more estrogen leads to a decrease in GM-CSF concentrations, while in males who have lower levels of estrogen, adding topical estrogen seems to increase its

concentrations. These data seem to correlate with tumor burden from past studies (data not shown) where intact female mice given additional topical estrogen demonstrated higher tumor burden than their control counterparts, while intact male mice given additional topical estrogen demonstrated a lower tumor burden relative to control mice. The potential for these changes in tumor burden to come as a result of estrogen's interactions with cytokines involved in immune cell maturation is very exciting.

Interleukin 16 (IL-16) is a cytokine known to act as a chemoattractant for cells that express CD4. These cells include monocytes, eosinophils, and dendritic cells, all involved in the immune system's inflammatory response to threats in the cellular environment [16]. Its nature as a chemoattractant makes information on its levels in the skin following irradiation interesting, as it serves as a marker of immune cell recruitment to affected areas. We see that, similar to the trends of GM-CSF, IL-16 was higher in control females treated with acetone relative to males of the same treatment group. In the context of our hypothesis, this could mean that females are better able to recruit immune cells at baseline relative to males, helping them rid the cellular environment of threats. Furthermore, in mice treated with topical estrogen we see that males and females have essentially the same levels of IL-16. Like in GM-CSF, females given topical estrogen had lower levels of IL-16 compared to control females, whereas estrogen treated males had higher levels compared to controls. Therefore, IL-16 levels may decrease when additional topical estrogen is added to intact females leading to more tumors through a reduction in immune cell recruitment. In males IL-16 levels may increase in response to estrogen leading to more immune cell recruitment and a lesser tumor burden. These

explanations seem plausible as they correlate with tumor burden data from past studies (data not shown).

sICAM-1 is soluble form of Intercellular Adhesion Molecule 1(ICAM-1). It can be stimulated by other inflammatory cytokines including Interleukin 1(IL-1) and TNF-a. Upon stimulation, leukocytes bind to the cells that express ICAM-1, allowing them to migrate from the blood stream into tissues [37, 38]. This function makes it an important mediator for immune cell migration into the skin as part of the inflammatory response to UV irradiation. In our studies, male control mice had higher levels of sICAM-1 relative to females in the same treatment group. These trends at first seem contradictory, however, research shows that a reduction in ICAM-1 actually increases the levels of other notable pro-inflammatory markers in the skin through some sort of compensatory mechanism [43].In light of this research, the lower levels of sICAM-1 in females may actually work to improve the inflammatory response to irradiation relative to the higher levels in males. In mice treated with topical estrogen, the levels of sICAM-1 drop to similar levels in both sexes compared to their acetone treated control counterparts, with males having a larger decrease. In the context of this research, this larger decrease perhaps leads to a larger compensatory response by other inflammatory markers, resulting in the lesser tumor burden seen in mice treated with topical estrogen. The lower levels in female mice treated with topical estrogen, relative to controls, is somewhat contradictory. If we assume that a decrease in ICAM-1 leads to more inflammation through compensatory mechanisms, it follows that there should be a decrease in tumor burden in female mice treated with topical estrogen. This demands further research, but it is possible that

intact females given additional topical estrogen have less compensatory inflammation, leading to a decrease in cutaneous immune cell infiltration along with a lesser inflammatory response.

Interleukin 1 Receptor Antagonist (IL-1ra) is, as the name suggests, an antagonist to the IL-1 receptor which prevents IL-1 signaling in cells [39]. IL-1 plays various roles in the initial activation of the inflammatory response which include inducing neutrophil localization, activating lymphocyte proliferation, and inducing fever [15]. The inhibition of these pro-inflammatory effects by IL-1ra within the cellular environment is of great interest. Inhibition of inflammation may lead to a reduction of pre-cancerous cell clearance and therefore greater tumor burden. In acetone treated control females, the levels of IL-1ra are lower than those of males in the same treatment group. This trend suggests that females have less inhibition of the IL-1 mediated inflammatory response than males at baseline. Greater levels of inflammation resultant from less inhibition have the potential to rid the body of more pre-cancerous cells, leading to the lesser tumor burden seen in female mice. Mice of both sexes treated with topical estrogen exhibit lower levels of IL-1ra than the acetone treated control mice, with males demonstrating a larger difference. It is possible that estrogen does indeed reduce the levels of IL-1ra antagonist in both sexes, but due to other interactions between estrogen and the inflammatory response, the pro-inflammatory effects that would normally accompany an increase in IL-1 binding are altered later in the inflammatory pathway.

In control mice, females demonstrated lower levels of IL-1a than males in the same treatment group. It was also seen that estrogen treated females had lower levels of the cytokine than control mice, while estrogen treated males had levels that were very slightly lower than controls. As previously mentioned, it is possible that estrogen elicits effects later on

in the inflammatory pathway that outweigh the actions of IL-1a in the initiation of inflammation. It is important to note that the levels of IL-1a need not correspond with the trends seen in the levels of IL-1ra, as the latter cytokine simply blocks the receptor for the former; IL-1ra does not actually alter the levels of IL-1a present.

Tumor Necrosis Factor alpha (TNF-a) is heavily involved in the inflammatory process. It is largely produced by macrophages during inflammation, and has actions that include acting as a chemoattractant for neutrophils and stimulating phagocytosis and the production of Prostaglandin E_2, an inflammatory lipid [13]. Our cytokine data showed that in control mice, females have higher levels of TNF-a relative to males. This trend between the sexes provides further evidence that females produce a more robust immune response, which may explain their lesser tumor burden. The levels of TNF-a in both sexes treated topically with estrogen remain extremely close to those in control mice. While tumor burden did differ between mice given topical estrogen and those that weren't, TNF-a levels did not. Based on this, we believe that differences in tumorigenesis between mice treated with topical estrogen and those that were not, are primarily mediated by a cytokine(s) further down the inflammatory pathway or by differences in immune cell levels. Within the same control group, however, differences in tumor burden are likely influenced by the levels of TNF-a and the inflammatory effects it mediates.

Even with some cytokines demonstrating trends that seem to contradict what would be expected given their roles in the inflammatory response, we believe that we have some exciting data. The extent to which each of the studied cytokines contributes to the sex differences in inflammatory response is not yet clear, and nor is the point(s) at which the link between

estrogen and inflammation exists. This exploratory series of cytokine arrays provided evidence of exciting trends where the levels of cytokines are shown to be effected by estrogen, and these trends correlate with tumor burden in the context of our hypothesis. With continued investigation we hope to validate trends that were explored here, implicating key inflammatory cytokines in the interplay between estrogen and inflammation. It is also our goal to complete chronic UV studies, where the levels of inflammatory cytokines, the effects estrogen has on them and the broad inflammatory response can be directly related to tumor burden within the same study. We have also recognized the potential for the involvement of estrogen receptors in the sex differences in inflammatory response. We plan to investigate the extent to which they may influence the immune system and inflammatory protein production, as well as the differences in their levels between the sexes.

References

1. Rogers, H.W., et al., *Incidence estimate of nonmelanoma skin cancer in the United States, 2006.* Arch Dermatol. 146(3): p. 283-7.

2. Stern, R.S., *Prevalence of a history of skin cancer in 2007: results of an incidence-based model.* Arch Dermatol. 146(3): p. 279-82.

3. Robinson, J.K., *Sun exposure, sun protection, and vitamin D.* JAMA, 2005. 294(12): p. 1541-3.

4. *Squamous Cell Carcinoma*. cited 2012; Available from: http://www.aad.org/skin-conditions/dermatology-a-to-z/squamous-cell-carcinoma.

5. Rogers, H.W., "*Your new study of nonmelanoma skin cancers.*", T.S.C. Foundtation, Editor. 2010.

6. Koh, H.K., et al., *Prevention and early detection strategies for melanoma and skin cancer. Current status.* Arch Dermatol, 1996. 132(4): p. 436-43.

7. Saladi, R.N. and A.N. Persaud, *The causes of skin cancer: a comprehensive review.* Drugs Today (Barc), 2005. 41(1): p. 37-53.

8. Thomas-Ahner, J.M., et al., *Gender differences in UVB-induced skin carcinogenesis, inflammation, and DNA damage.* Cancer Res, 2007. 67(7): p. 3468-74.

9. Wilgus, T.A., et al., *Inhibition of cutaneous ultraviolet light B-mediated inflammation and tumor formation with topical celecoxib treatment.* Mol Carcinog, 2003. 38(2): p. 49-58.

10. Wilgus, T.A., et al., *Chemotherapeutic efficacy of topical celecoxib in a murine model of ultraviolet light B-induced skin cancer.* Mol Carcinog, 2003. 38(1): p. 33-9.

11. Brenner, Michaela, and Vincent J. Hearing. “The Protective Role of Melanin Against UV Damage in Human Skin.” *Photochemistry and photobiology* 84.3 (2008): 539–549. *PMC*. Web. 10 Apr. 2015.

12. Mayer, G., *Microbiology and Immunology On-Line Text Book - Chapter One: Innate Immunity*. 2006: USC School of Medicine.

13. Gahring, L. C., N. G. Carlson, R. A. Kulmar, and S. W. Rogers. "Neuronal Expresseion of Tumor Necrosis Factor Alpha in the Murine Brain." Neuroimmunomodulation 3.5 (1996): 289-303. Web.

14. Ferguson-Smith AC, Chen YF, Newman MS, May LT, Sehgal PB, Ruddle FH (April 1988). "Regional localization of the interferon-beta 2/B-cell stimulatory factor 2/hepatocyte stimulating factor gene to human chromosome 7p15-p21". Genomics 2 (3): 203–8.

15. Dinarello CA (2011). "Interleukin-1 in the pathogenesis and treatment of inflammatory diseases". Blood 117 (14): 3720–32.

16. "Genes and Mapped Phenotypes." National Center for Biotechnology Information. U.S. National Library of Medicine, n.d. Web. 10 Apr. 2015.

17. Cotran; Kumar, Collins (1998). Robbins Pathologic Basis of Disease. Philadelphia: W.B Saunders Company

18. Witko-Sarsat, V; Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000). "Neutrophils: molecules, functions and pathophysiological aspects". Lab Invest 80 (5): 617–53.

19. Coussens, Lisa M., and Zena Werb. "Inflammation and cancer." Nature 420.6917 (2002): 860-867.

20. Haabeth, Ole Audun Werner, et al. "Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer." *Nature communications* 2 (2011): 240.

21. Rogers, HW, Weinstock, MA, Harris, AR, et al. Incidence estimate of nonmelanoma skin cancer in the United States, 2006. Arch Dermatol 2010; 146(3):283-28

22. American Cancer Society. Cancer Facts & Figures 2015. http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-044552.pdf.

23. Sullivan, N.J., et al., UV light B-mediated inhibition of skin catalase activity promotes Gr-1+ CD11b+ myeloid cell expansion. J Invest Dermatol. 132(3 Pt 1): p. 695-702.

24. Cohen, C.A., et al., Intra-abdominal fat depots represent distinct immunomodulatory microenvironments: a murine model. PloS ONE. 8(6): p. e66477.

25. Ogden, C.L.K., B.K. Flegal, K.M., Prevalence of Obesity in the United States, 2009-2010. 2012, CDC.

26. Garofalo, C. and E. Surmacz, *Leptin and cancer.* J Cell Physiol, 2006. 207(1): p. 12-22.

27. van Kruijsdijk, R.C., E. van der Wall, and F.L. Visseren, *Obesity and cancer: the role of dysfunctional adipose tissue.* Cancer Epidemiol Biomarkers Prev, 2009. 18(10): p. 2569-78.

28. "Genes and Mapped Phenotypes." National Center for Biotechnology Information. U.S. National Library of Medicine, n.d. Web. 10 Apr. 2015.

29. Stanworth, Roger D, and T Hugh Jones. “Testosterone for the Aging Male; Current Evidence and Recommended Practice .” Clinical Interventions in Aging 3.1 (2008): 25–44.

30. Reeve, Vivienne E., et al. "Protection against photoaging in the hairless mouse by the isoflavone equol." Photochemistry and photobiology 81.6 (2005): 1548-1553.

31. Ormerod, Anthony David, et al. "The inflammatory and cytotoxic effects of a nitric oxide releasing cream on normal skin." Journal of investigative dermatology 113.3 (1999): 392-397.

32. Pendás, Alberto M., et al. "Diet-induced obesity and reduced skin cancer susceptibility in matrix metalloproteinase 19-deficient mice." Molecular and cellular biology 24.12 (2004): 5304-5313.

33. Manthey HD, Woodruff TM, Taylor SM, Monk PN (2009). "Complement component 5a (C5a).". Int J Biochem Cell Biol 41 (11): 2114–7.

34. Francisco-Cruz A, Aguilar-Santelises M, Ramos-Espinosa O, Mata-Espinosa D, Marquina-Castillo B, Barrios-Payan J et al. (Jan 2014). "Granulocyte-macrophage colony-stimulating factor: not just another haematopoietic growth factor". Medical Oncology 31 (1): 774.

35. J. Immunol. 157:4664-4671, 1996

36. Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y, Yeung YG (January 1997). "Biology and action of colony--stimulating factor-1". Mol. Reprod. Dev. 46 (1): 4–10.

37. Abe Y, El-Masri B, Kimball KT et al. (May 1998). "Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion". Arterioscler. Thromb. Vasc. Biol. 18 (5): 723–31.

38. Rothlein, R. D.; Dustin, M. L.; Marlin, S. D.; Springer, T. A. (Aug 1986). "A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1". *Journal of Immunology* 137 (4): 1270–1274.

39. Perrier S, Darakhshan F, Hajduch E (November 2006). "IL-1 receptor antagonist in metabolic diseases: Dr Jekyll or Mr Hyde?". FEBS Lett. 580 (27): 6289–94.

40. Skurk, Thomas, et al. "Relationship between adipocyte size and adipokine expression and secretion." The Journal of Clinical Endocrinology & Metabolism 92.3 (2007): 1023-1033.

41. Ramos-Vara, JA; Miller MA (2014). "When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique.". Veterinary Pathology 51 (1): 42–87.

42. Bates, Richard R. "Sex Hormones and Skin Tumorigenesis. I. Effect of the Estrous Cycle and Castration on Tumorigenesis by 7, 12-dimethylbenz [a] anthracene." Journal of the National Cancer Institute 41.2 (1968): 559-563.

43. Mitsui, Aya, et al. "Deficiency of both L-selectin and ICAM-1 exacerbates imiquimod-induced psoriasis-like skin inflammation through increased infiltration of antigen presenting cells." Clinical Immunology (2015).